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13. ABSTRACT ***(Maximum 200 Words)***

The goal of this research was to develop a technique for imaging living human breast epithelial cells in 3-D cultures and to evaluate their responses to the application of exogenous factors. Most model systems used to study breast cancer utilize cells growing in monolayers on plastic substrates. Although a great deal of information about cells and their responses to exogenous agents, such as therapeutic drugs, can be learned from these studies, there are also major limitations to this approach. In short, cells growing on plastic are flat, whereas cells in the body are three-dimensional. Recent data from a number of laboratories demonstrate that cells growing in monolayers do not necessarily respond to exogenous substances in the same manner as cells growing in 3-D (Weaver et al., 1997). Therefore, we developed technology for imaging human mammary epithelial cells growing in a three-dimensional reconstituted basement membrane. This technique enables monitoring fluorescently labeled proteins in living "normal," premalignant and tumor cells. This approach can be used to detect rapid, "real-time" responses by these cells to the effects of a spectrum of exogenous factors, including therapeutic agents.

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INTRODUCTION

The goal of this research was to develop a technique for imaging living human breast epithelial cells in 3-D cultures and to evaluate their responses to the application of exogenous factors. Most model systems used to study breast cancer utilize cells growing in monolayers on plastic substrates. Although a great deal of information about cells and their responses to exogenous agents, such as therapeutic drugs, can be learned from these studies, there are also major limitations to this approach. In short, cells growing on plastic are flat, whereas cells in the body are three-dimensional. Recent data from a number of laboratories demonstrate that cells growing in monolayers do not necessarily respond to exogenous substances in the same manner as cells growing in 3-D (Weaver et al., 1997). Therefore, we developed technology for imaging human mammary epithelial cells growing in a three-dimensional reconstituted basement membrane. This technique enables monitoring fluorescently labeled proteins in living "normal," premalignant and tumor cells. This approach can be used to detect rapid, "real-time" responses by these cells to the effects of a spectrum of exogenous factors, including therapeutic agents.

BODY

Specific Aim 1: Visualization of Living Human Mammary Epithelial Cells Growing in a Three-Dimensional Matrix

Tracking proteins in live human mammary epithelial cells growing in three-dimensional reconstituted basement membrane components (i.e. Matrigel) is more complex than tracking them in two-dimensional cultures. As we discussed in our previous report, the use of confocal microscopy solved some of the optical problems involved in this process, making it feasible to examine specific organelles labeled with vital dyes in living cells. However, tracking proteins tagged with the green fluorescent protein (GFP) introduced a number of additional problems. Examining fluorescent proteins in living cells can be problematic whether the cells are growing in 2-D or 3-D cultures. The lasers utilized to excite the fluorophore can themselves damage the cells and excitation of the fluorophore can also generate photoxidants that are toxic to the cells. Extreme caution must be used for these studies to assure that one is studying a healthy cell. The best approach currently available for examining fluorescent proteins in thick (100 to 200 micron) cultures is multi-photon microscopy. We purchased the Zeiss 510 NLO system, which was installed in October 2000. This solved many of the problems we had encountered (One still must be cautious! New problems associated with IR excitation, such as excessive heat, can occur.) We were then able to examine the protein beta-catenin, as outlined in the proposal, in living cells.

Tracking the GFP protein construct of beta-catenin also turned out to be problematic. As discussed in our previous report, cells examined at early time points after transfection with beta-catenin-GFP demonstrated the presence of small foci of beta-catenin in the nucleus. At later time points, large clusters of beta-catenin-GFP were seen

in the nucleus of the cells, but the cells had become rounded and demonstrated several projections from the cell surface that extended and retracted at multiple sites around the cell. Additional studies since have demonstrated that all cells transfected with beta-catenin-GFP undergo apoptosis. This has been confirmed both biochemically and using imaging techniques (See Figures 1 and 2). These results are specific to transfection with the protein beta-catenin, since transfection with either the empty GFP vector or actin-GFP failed to induce apoptosis. An abstract reporting these results has been submitted to a Keystone symposium on Wnt and Beta-Catenin Signaling in Development and Disease to be held in March 2002, and a manuscript is in preparation.

Specific Aim #2: Examination of the Effects of Exogenous Factors on Living Human Mammary Epithelial Cells Growing in a Three-Dimensional Matrix.

Metastasis is a major problem associated with cancer and is related to the ability of cells to migrate. By examining live cells that had been transfected with actin-GFP (normal and tumor cells) using time-lapse imaging, the increased motility and migratory behaviors of the tumor cells became quite evident. Unfortunately, the ability to document and quantify these behaviors in living cells is limited. Existing techniques involve time-lapse videos of a limited number of cells (Rajah et al., 1998), which makes it difficult to obtain statistically significant studies of cell populations. Improved statistics can be obtained with the "scratched wound method" (Bürk, 1981; Környei et al, 2000), in which a region of the cell culture substrate is denuded of cells and then the time scale for the filling of this "hole" is observed. Unfortunately, the history of the cell migration paths is lost, and the analysis is complicated by subjective analysis of the complex and variable patterns of cell motion that lead to hole filling. A significant advance occurred with the development of the Boyden Chamber invasion assay, in which cells are seeded on one side of a membrane, and the number of cells reaching the other side is determined (Boyden, 1962; Yao et al., 1990). This method, though widely used, is extremely laborious and requires that the cells be fixed, and thus destroyed, preventing real time variation of the external conditions.

In collaboration with Prof. Paul Alivisatos, a chemist at the University of California at Berkeley, we developed a technique to use colloidal quantum dots to monitor cell motility. These quantum dots are protein-sized crystals of inorganic semiconductors that are robust and efficient light emitters (Bawendi, et al., 1990; Alivisatos, 1996). When coated with a suitable solubilizing layer, such as silica, they are stable under physiological buffer conditions (Gerion et al., 2001). We showed that the colloidal semiconductor nanocrystals are ingested by a wide variety of cells, while remaining fully luminescent, thus enabling the tracking of dynamical phenomena inside cells over periods of weeks. In addition, we demonstrated that this property can be used for quantum dot based imaging of phagokinetic tracks (see Appendix for manuscript). Additional experiments not included in our submitted manuscript are in progress to demonstrate the ability to use the quantum dots as an assay for examining cell motility and invasiveness in living cells growing in 3-D cultures. The use of the quantum dots also provides an excellent assay for the effects of exogenous factors on living human

mammary epithelial cells growing in both two and three-dimensional cultures. (A patent on this technique has been filed; see attached Report of Inventions and Subcontracts).

In another set of experiments, we used this assay to demonstrate the ability of a peptide obtained from a plant to block cell motility (manuscript in preparation; see Figure 3). The peptide, referred to as SPIKE, bundles actin. It was isolated from a plant protein by Drs. Steven Huber and Heike Winter, collaborators from North Carolina State University. In another set of experiments in which we injected the peptide into *Xenopus laevis* eggs shortly after fertilization, we demonstrated the ability of this peptide to block cell division as well. Embryos injected with the control, inactive peptide cleaved normally and were fixed at the 8-cell stage. Those embryos injected with SPIKE failed to complete cleavage. Cleavage furrows were formed, but appear to become "fixed," preventing complete cell division and blastomere formation. Labeling with rhodamine phalloidin, which binds filamentous actin, demonstrates the presence of increased amounts of actin in the abortive cleavage furrows.

KEY RESEARCH ACCOMLISHMENTS:

- Demonstrated the ability to track protein constructs tagged with green fluorescent protein (GFP) in three-dimensional matrix (Matrigel)
- Demonstrated that over-expression of beta-catenin in human mammary epithelial tumor cells (MCF-7) induces apoptosis.
- Developed fluorescence-based assay using colloidal quantum dots to measure cell motility and migration in living cells growing in two- and three-dimensional cultures.
- Used the quantum dot assay to demonstrate the ability of a peptide derived from a plant protein to block cell motility and cytokinesis.

REPORTABLE OUTCOMES

- **Manuscripts**
  - Parak, W.J., Boudreau, R., Le Gros, M.A., Gerion, D., Zanchet, D., Micheel, C.M., Williams, S.C., Alivisatos, A.P. and Larabell, C.A. (2001) Cell Motility and Metastatic Potential Studies Based on Quantum Dot Imaging of Phagokinetic Tracks. *Advanced Materials*. Submitted.
  - Boudreau, R., Engel, B., Le Gros, M.A., and Larabell, C.A. Overexpression of ß-catenin Promotes Apoptosis in Human Mammary Epithelial Tumor Cells. Manuscript In Preparation.
  - Winter, H., Boudreau, R., Holtgraewe, D., Huber, S., and Larabell, C.A. Reorganisation of actin cytoskeleton by SPIKE blocks cell motility and inhibits cytokinesis. Manuscript in Preparation.

- **Presentations**
  - "Tracking GFP-tagged proteins in human mammary epithelial cells in culture." Poster, Era of Hope, June 2000.
  - "Measuring cell motility and metastatic potential with quantum dots." Gordon Conference, *Lasers in Medicine and Biology.* July 2002.

- **Patents**
  - "Cellular Imaging Using Semiconductor Nanocrystals," IB-1755P. Carolyn A. Larabell, Mark Le Gros, Rosanne Boudreau, Wolfgang J. Parak, A. Paul Alivisatos.

- **Invention Disclosure**
  - "A synthetic peptide that causes bundling of filamentous actin (F-actin) in vitro and in situ," JIB-1571. Steven C. Huber, Heike Inge Ada Winter, Carolyn A. Larabell

CONCLUSIONS

Imaging living cells reveals data that could not readily be obtained using other techniques. We are developing techniques for imaging human mammary epithelial cells growing in 3-D cultures and for tracking GFP protein constructs in these cells. We expect this approach will yield valuable information about the responses of tumor cells to exogenous agents that would not otherwise be generated from studying fixed specimens. The use of new imaging techniques, specifically the multi-photon microscope, has enabled us to conduct experiments on cells growing in thick (up to 200 microns), three-dimensional matrices. However, our attempt to study the protein beta-catenin in normal and tumor cells by introducing a GFP construct of this protein into cells led to an entirely unexpected outcome. Over-expression of beta-catenin induced apoptosis in the cells. This, in itself, is an interesting and reportable outcome of the experiments. But it also demonstrates the hazards of studying exogenous proteins, especially those with signaling capacity; over-expression of the protein one is attempting to study may actually alter the behavior of the cells.

We were very successful in developing an assay for monitoring the effects of exogenous factors on living human mammary epithelial cells growing in two- and three-dimensional cultures. In collaboration with Prof. Paul Alivisatos, a chemist at the University of California at Berkeley, we developed a fluorescence-based assay that uses colloidal quantum dots to monitor cell motility and migration and, when applied to three-dimensional extracellular matrices, can be used to measure invasiveness. The strengths of this assay are: 1) It can be used with living cells; 2) It allows monitoring changes in cell behaviors in response to addition of exogenous agents; 3) There is no processing required, eliminating loss of cells and making it more accurate; and 4) It can be quantitative. We anticipate this will be an extremely useful assay for cell biologists studying cell motility as well as for cancer biologists.

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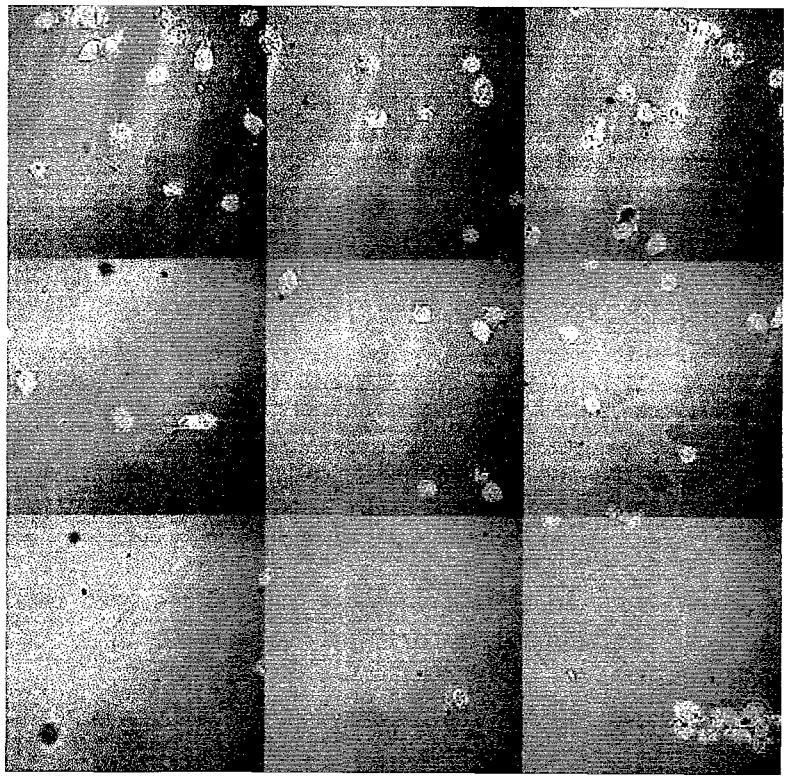
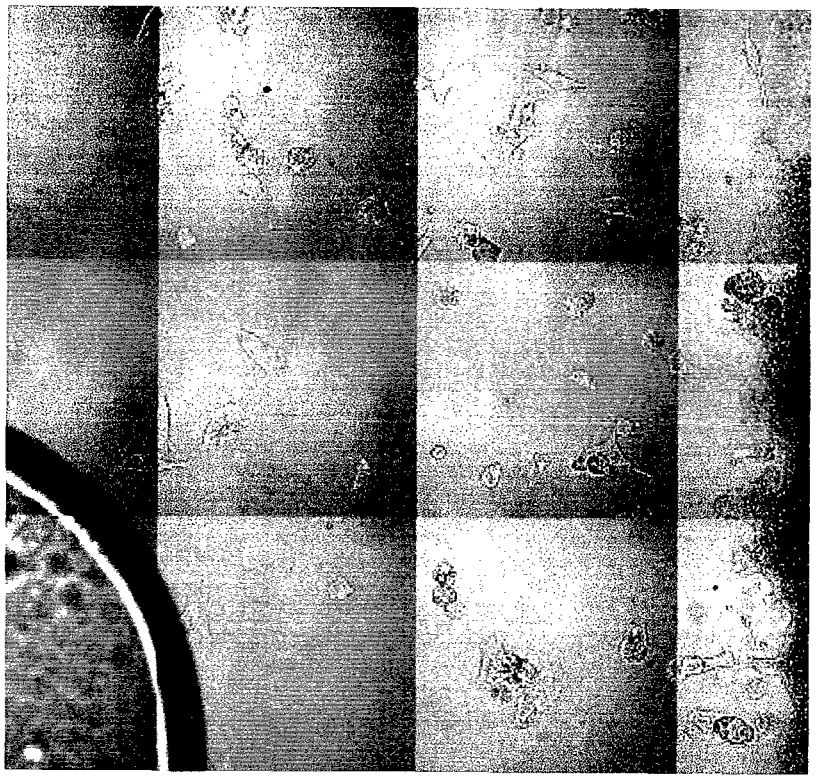
Figure 1

MCF 7 Cells Transfected With Beta-Catenin-GFP Undergo Apoptosis



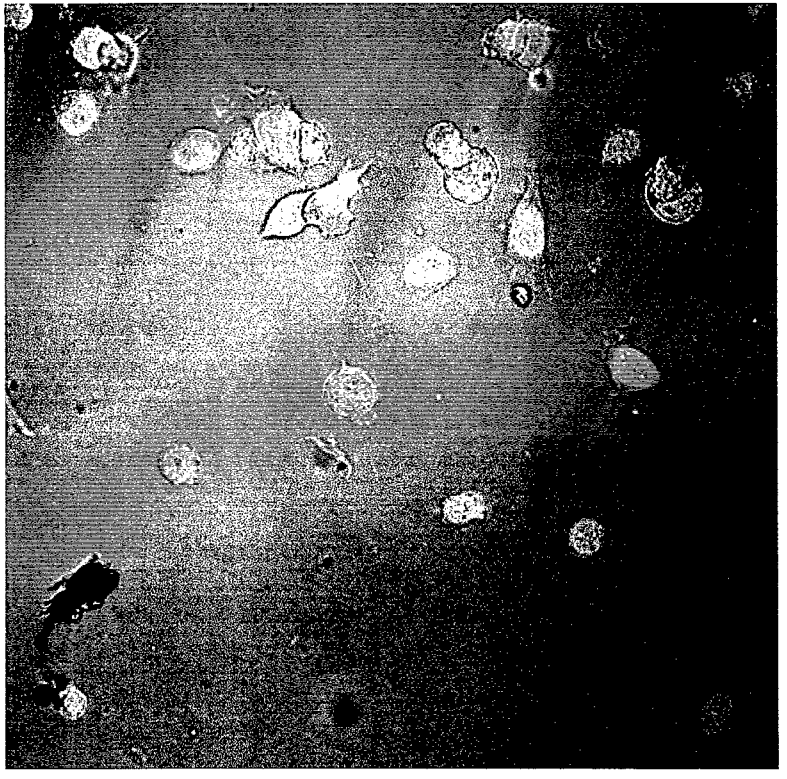
Transfected
Apo-tag positive



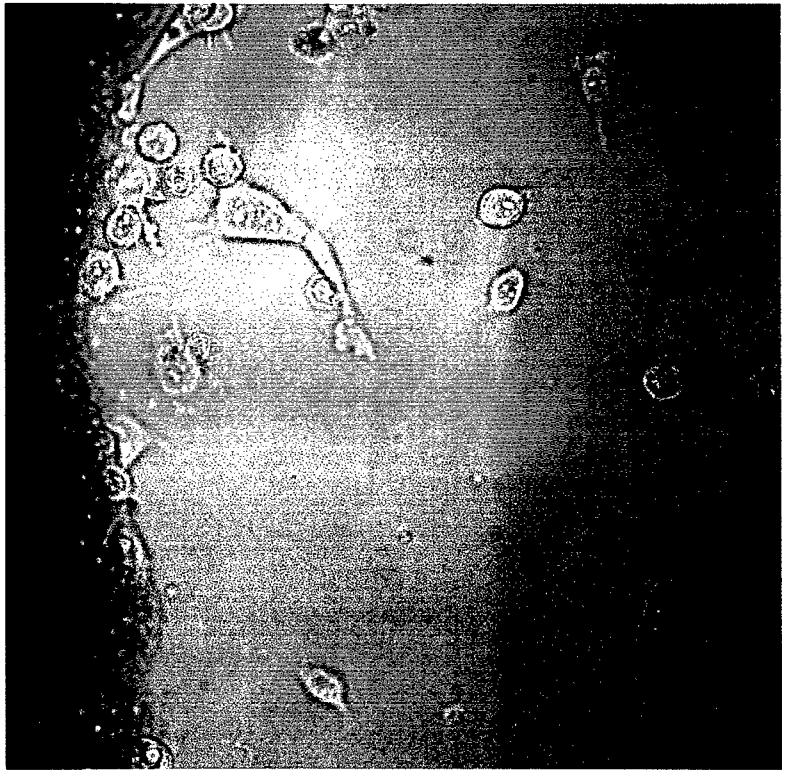
Not transfected
Apo-tag negative

Figure 2

MCF 7 Cells Transfected With Beta-Catenin-GFP Undergo Apoptosis



Transfected
Apo-tag positive



Transfected
Control (NSB)

Figure 3

Effect of Synthetic Peptide on Cleavage of Xenopus Embryos

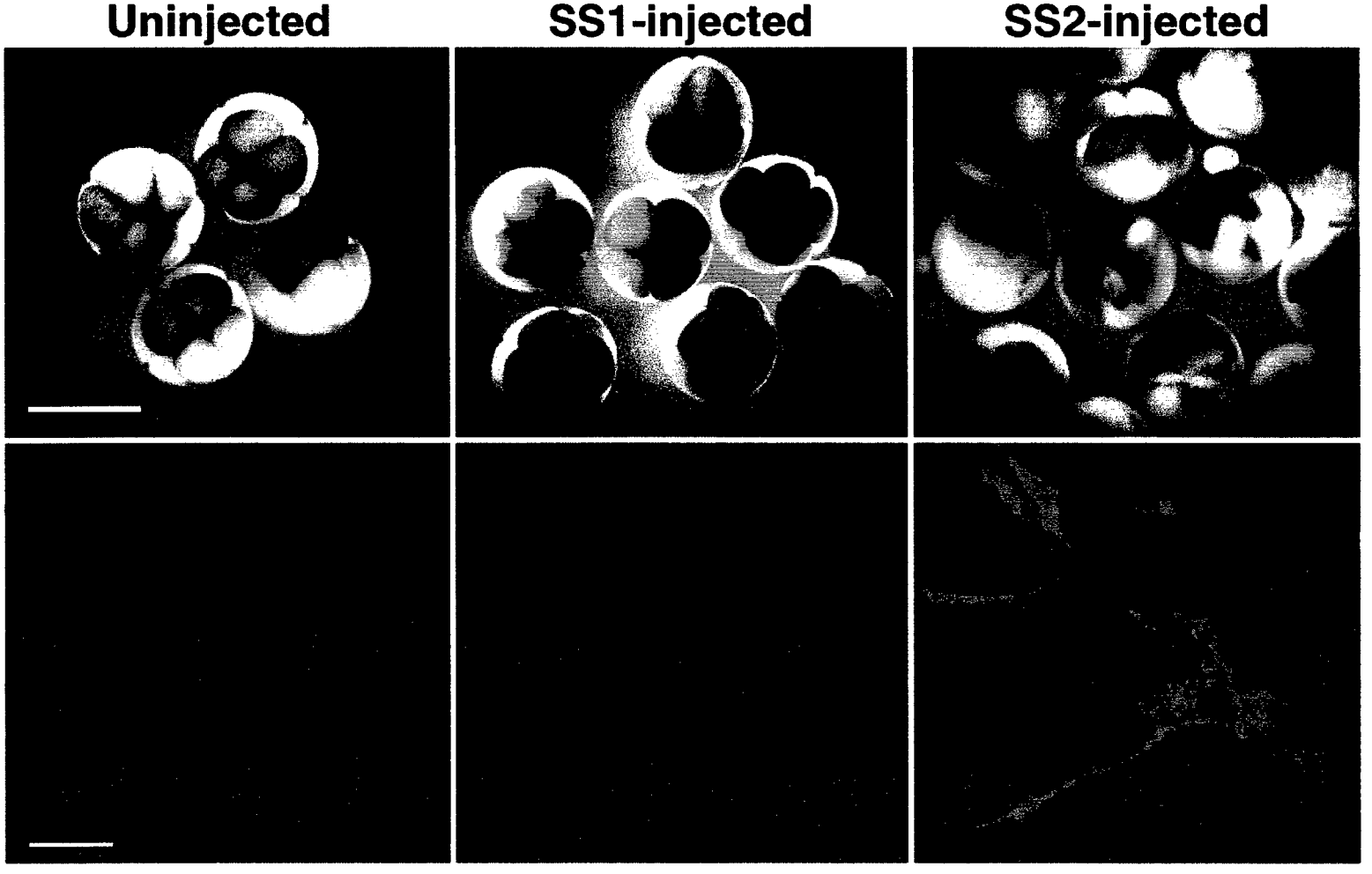
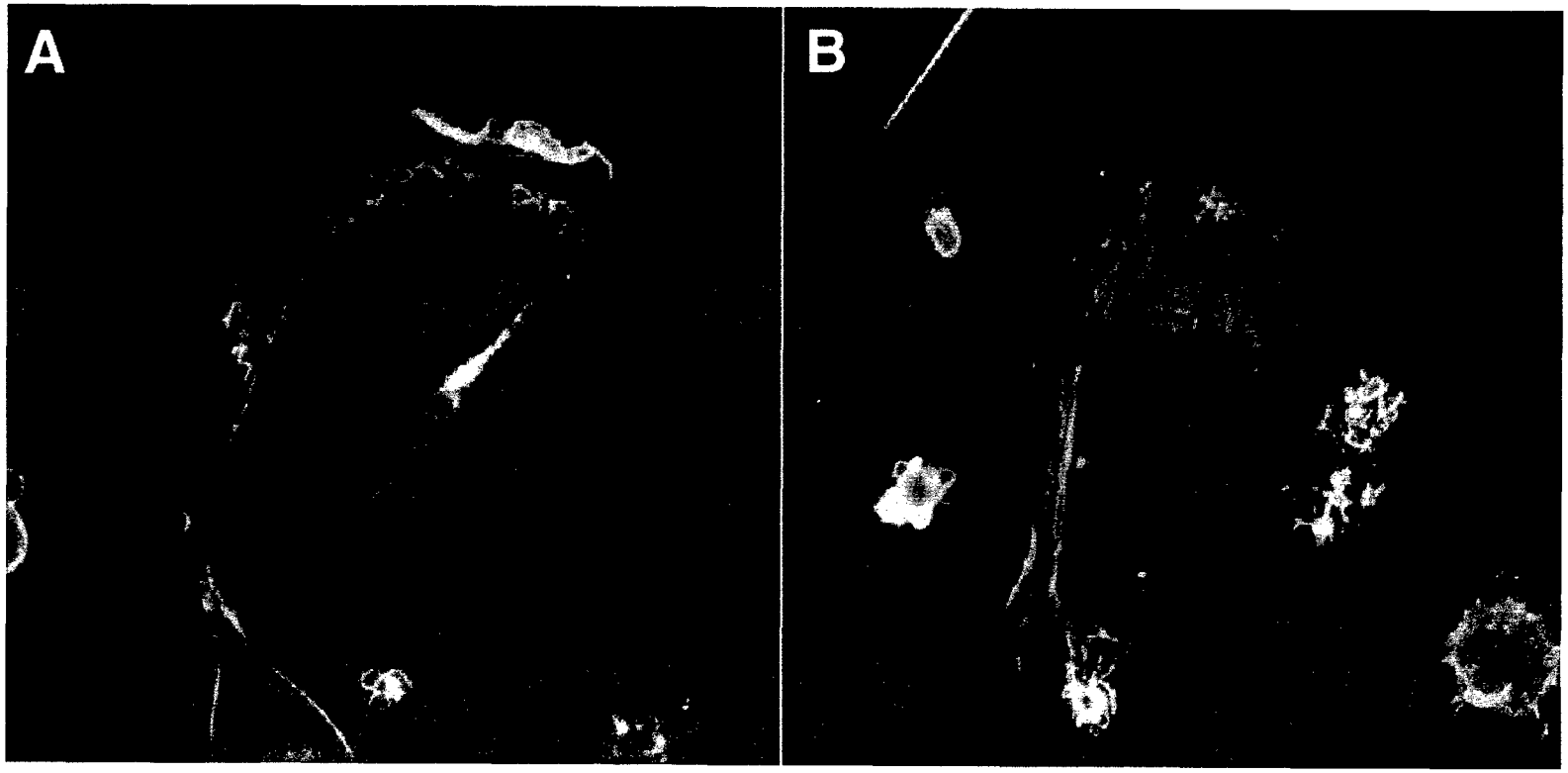


Figure 4

Treatment of Tumor Cells with Plant Compound that Stops Cell Crawling



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